This is the post print version of the article, which has been published in Bioorganic and medicinal chemistry . 2018, 26 (14), 4187-4190. https://doi.org/10.1016/j.bmc.2018.07.011.

Activation studies with amines and amino acids of the α-carbonic anhydrase from the pathogenic protozoan *Trypanosoma cruzi*

Andrea Angeli,[a] Marianne Kuuslahti,[b] Seppo Parkkila[b] and Claudiu T. Supuran[a]*

[a]Università degli Studi di Firenze, Dipartimento Neurofarba, Sezione di Scienze Farmaceutiche e Nutraceutiche, Via U. Schiff 6, 50019 Sesto Fiorentino, Florence, Italy.

[b] Faculty of Medicine and Life Sciences, University of Tampere; Fimlab Ltd., Tampere University Hospital, 33520 Tampere, Finland.

Abstract. The activation of a α-class carbonic anhydrase (CAs, EC 4.2.1.1) from *Trypanosoma cruzi* (TcCA) was investigated

Keywords: carbonic anhydrase; metalloenzymes, pathogens; activators; *Trypanosoma cruzi*

*Corresponding authors: Tel/Fax: +39-055-4573729, E-mail: claudiu.supuran@unifi.it (Claudiu T. Supuran).

1. Introduction

Protozoan carbonic anhydrases (CAs, EC 4.2.1.1) such as those from *Trypanosoma cruzi*, [1-3] *Leishmania donovani chagasi*,[1,4,5] and *Plasmodium falciparum*,[4,6] started to be investigated in recent years as potential drug targets for finding agents enzymes that interfere with the growth and proliferation of the parasites which provoke widespread diseases all over the world.[2-11] *T. cruzi* encodes for an α-class [7,8] CA (TcCA), which has recently been cloned, characterized and investigated for its inhibition, being shown to be a promising new target in the fight against Chagas disease, provoked after infection with this protozoan species.[1-3] Indeed, both sulfonamide, thiol or hydroxamate CA inhibitors (CAIs)7-9 were shown to effectively inhibit *in vitro* this enzyme, and in some cases, also to interfere with the growth of some forms of the parasite in vivo, which may lead to the discovery of potential drugs devoid of the drug resistance problems encountered by the few clinically used compounds available so far for the treatment of Chagas disease.[1-3]

However, in contrast to the CAIs, which were extensively investigated for their interaction with various protozoan CAs. Such as those from the malaria parasite, *Leishmania* spp., and *T cruzi*,[1] the CA activators (CAAs)[10-12] have been much less investigated at the present time. For the mammalian CA isoforms, also belonging to the α-CA genetic family,[13-16] similar to TcCA, it has been shown that such compounds participate to the CA catalytic cycle, which is shown schematically in Equations 1 and 2 (where 'E' denotes enzyme):

$$EZn^{2+}\text{—}OH^- + CO_2 \Leftrightarrow EZn^{2+}\text{—}HCO_3^- \Leftrightarrow EZn^{2+}\text{—}\overset{H_2O}{OH_2} + HCO_3^- \quad (1)$$

$$EZn^{2+}\text{—}OH_2 \Leftrightarrow EZn^{2+}\text{—}OH^- + H^+ \quad (2)$$

The first step (Equation 1) involves a nucleophilic attack of a zinc-bound hydroxide species of the enzyme on the CO_2 substrate, that is bound in a hydrophobic pocket nearby. in an optimal orientation for the hydration reaction to bicarbonate.[11,16] Bicarbonate formed in the hydration reaction is then replaced by an incoming water molecule to generate the catalytically acidic form of the enzyme, $EZn^{2+}\text{—}OH_2$ (Equation 1), whereas bicarbonate is released into the solution. For the regeneration of the zinc hydroxide species, a proton transfer reaction occurs from the Zn(II)-bound water molecule to the external reaction medium (Equation 2), which is the rate-determining step of the entire catalytic cycle.[10]

$$EZn^{2+}\text{—}OH_2 + A \Leftrightarrow [EZn^{2+}\text{—}OH_2 - A] \Leftrightarrow [EZn^{2+}\text{—}OH^- - AH^+] \Leftrightarrow EZn^{2+}\text{—}OH^- + AH^+ \quad (3)$$

enzyme - activator complexes

In the presence of activators (A in Equation 3), formation of enzyme-activator complexes occurs, in which the proton transfer step becomes intramolecular and thus, more efficient than the

corresponding intermolecular process shown schematically in Eq. 2.[10,16] The CA activation mechanism was demonstrated by extensive kinetic and X-ray crystallographic studies on the human isoforms hCA I and II.[10-16] Based on such studies it has been shown that the activator was bound at the entrance to the active site cavity, region from which it can interfere with the proton transfer reactions between the active site and the reaction medium. In fact, most of the activators studies so far belong to the amino and/or amino acid derivatives, and possess moieties with an appropriate pK_a (generally in the range of 6-8) for an efficient proton shuttling processes between the active site and the environment.[10-17]

CAAs were only recently shown to possess the potential of acting as pharmacological agents for the therapy of memory disorder and cognition impairment.[16] However, unlike CAIs, which are clinically used as diuretics,[18] antiglaucoma drugs,[19] antiobesity,[20] antitumor,[21] anti-neuropathic pain,[22] or anti-arthritis agents,[23] there are no clinically approved CAAs. The natural and non-natural amino acids and amines of type **1-24** are among the most investigated CAAs, and they were also evaluated in the present study for their interaction with the protozoan enzyme TcCA (Fig. 1).

2. Experimental

2.1. Chemistry. Amino acids and amines **1-24** were commercially available, highest purity reagents from Sigma-Aldrich, Milan, Italy. TcCA was a recombinant protein produced as reported earlier by our group.[2]

2.2. CA enzyme activation assay

An Sx.18Mv-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic activity of various CA isozymes for CO_2 hydration reaction.17 Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s at 25 ◦C. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and activation constants. For each activator at least six traces of the initial 5-10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of activators (10 mM) were prepared in distilled-deionized water and dilutions up to 1 nM were done thereafter with the assay buffer. Activator and enzyme solutions were pre-incubated together for 15 min (standard assay at room temperature) prior to assay, in order to allow for the formation of the E–A complex. The

activation constant (K_A), defined similarly with the inhibition constant K_I, can be obtained by considering the classical Michaelis–Menten equation (equation 4), which has been fitted by non-linear least squares by using PRISM 3:

$$v = v_{max}/\{1+(K_M/[S])(1+[A]_f/K_A)\} \quad (4)$$

where $[A]_f$ is the free concentration of activator.

Working at substrate concentrations considerably lower than K_M ($[S] << K_M$), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$)and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by equation 5:

$$v=v_0.K_A/\{K_A+([A]_t-0.5\{([A]_t+[E]_t+K_A)-([A]_t+[E]_t+K_A)^2-4[A]_t.[E]_t)^{1/2}\}\} \quad (5)$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[10,24,25]

3. Results and Discussion

Table 1: Activation of human carbonic anhydrase (hCA) isozymes I, II, and TcCA with L-Trp, at 25°C, for the CO_2 hydration reaction.[17]

Isozyme	k_{cat}* (s^{-1})	K_M* (mM)	$(k_{cat})_{L\text{-}Trp}$** (s^{-1})	K_A*** (μM) L-Trp
hCA I[a]	$2.0x10^5$	4.0	$3.4x10^5$	44.0
hCA II[a]	$1.4x10^6$	9.3	$4.9x10^6$	27.0
TcCA[b]	1.2×10^6	8.1	7.8×10^6	2.54

* Observed catalytic rate without activator. K_M values in the presence and the absence of activators were the same for the various CAs (data not shown).

** Observed catalytic rate in the presence of 10 μM activator.

*** The activation constant (K_A) for each enzyme was obtained by fitting the observed catalytic enhancements as a function of the activator concentration.[13] Mean from at least three determinations by a stopped-flow, CO_2 hydrase method.[30] Standard errors were in the range of 5-10 % of the reported values (data not shown).

[a]Human recombinant isozymes, from ref.[13]; [b] Protozoan recombinant enzyme, this work.

The structure-activity relationship (SAR) for the activation of TcCA with compounds **1-24**, can be delineated considering the data shown in Table 2, where the activation data of the human isoforms hCA I and II are also presented for comparison.

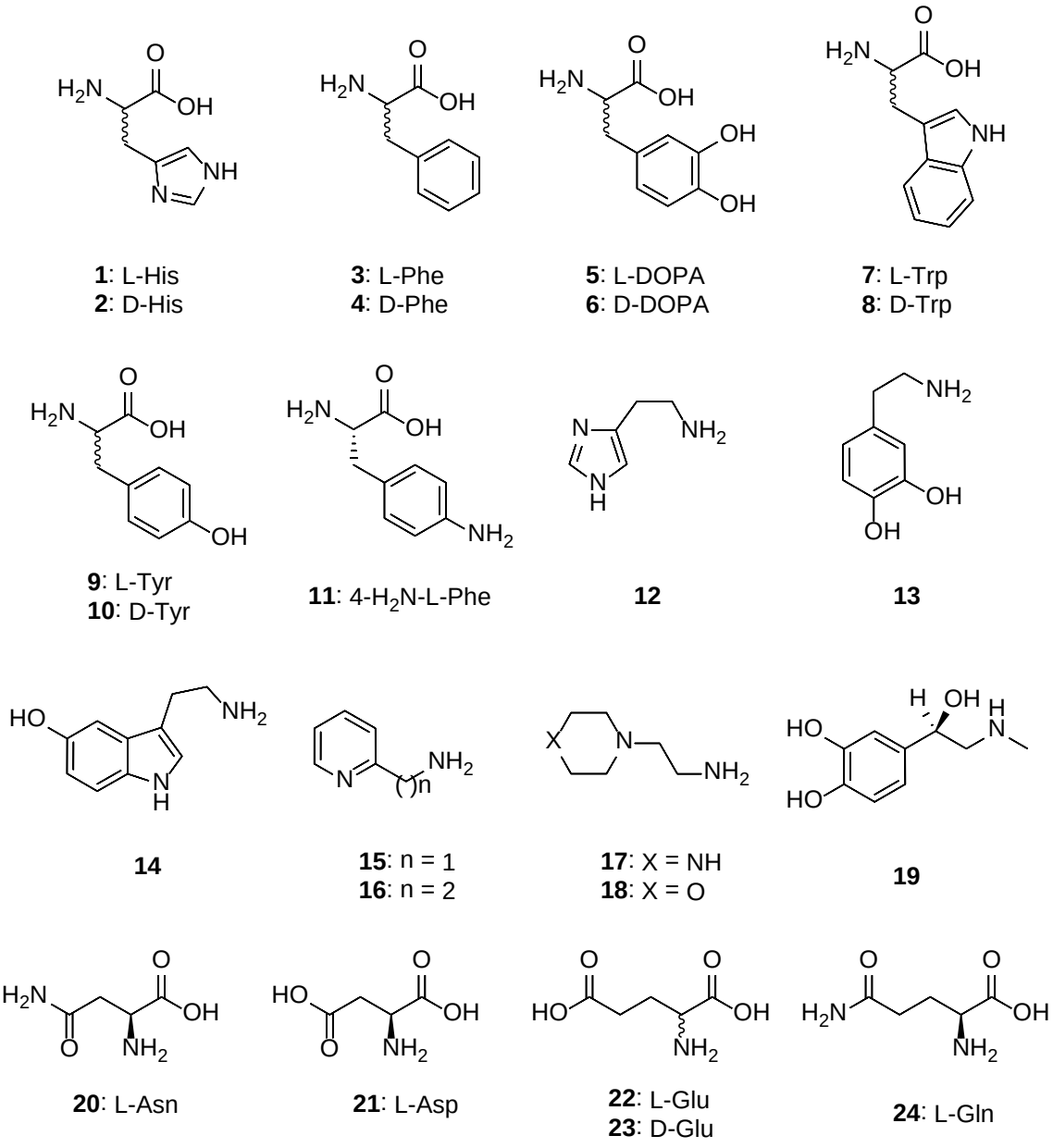

Fig. 1: Amino acids **1-24** investigated as TcCA activators.

Table 2: Activation constants of hCA I, hCA II and the protozoan enzyme TcCA with amino acids and amines **1 – 24**. Data for hCA I and II are from ref.[16]

No.	Compound	K_A (μM)*

		hCA I[a]	hCA II[a]	LdcCA[b]	TcCA[c]
1	L-His	0.03	10.9	8.21	11.3
2	D-His	0.09	43	4.13	7.54
3	L-Phe	0.07	0.013	9.16	12.1
4	D-Phe	86	0.035	3.95	6.39
5	L-DOPA	3.1	11.4	1.64	0.83
6	D-DOPA	4.9	7.8	5.47	0.38
7	L-Trp	44	27	4.02	2.54
8	D-Trp	41	12	6.18	1.79
9	L-Tyr	0.02	0.011	8.05	4.92
10	D-Tyr	0.04	0.013	1.27	2.80
11	4-H_2N-L-Phe	0.24	0.15	15.9	0.75
12	Histamine	2.1	125	0.74	2.73
13	Dopamine	13.5	9.2	0.81	>100
14	Serotonin	45	50	0.62	1.98
15	2-Pyridyl-methylamine	26	34	0.23	>100
16	2-(2-Aminoethyl)pyridine	13	15	0.012	>100
17	1-(2-Aminoethyl)-piperazine	7.4	2.3	0.009	>100
18	4-(2-Aminoethyl)-morpholine	0.14	0.19	0.94	6.95
19	L-Adrenaline	0.09	96	4.89	>100
20	L-Asn	11.3	>100	4.76	>100
21	L-Asp	5.20	>100	0.30	18.7
22	L-Glu	6.43	>100	12.9	>100
23	D-Glu	10.7	>100	0.082	>100
24	L-Gln	>100	>50	2.51	2.85

* Mean from three determinations by a stopped-flow, CO_2 hydrase method.[17] Standard errors were in the range of 5-10 % of the reported values (data not shown).

[a] Human recombinant isozymes, stopped flow CO_2 hydrase assay method;[16b]

[b] Protozoan recombinant enzyme, from ref. [25b]

[c] This work.

and of 13-15 μM for the human CAs. Thus, this compound may be used as a pharmacologic tool to explore the role that LdcCA might play in the life cycle of this protozoan and whether CA activation is important for the infection or host colonization by *Leishmania*, in diverse phases of the pathogen's life cycle.

4. Conclusions

The first activation study of a protozoan

Because activators have not been identified for protozoan CAs, this study should be important for understanding the role that this enzyme has in the life cycle of *Leishmania*, particularly considering the fact that many of the activators identified are autacoids present in rather high concentrations in different tissues of the host mammals that are infected by these parasites.

Acknowledgments. This research was financed in part by the Academy of Finland and Sigrid Juselius Foundation.

References

1. a) Vermelho AB, Capaci GR, Rodrigues IA, Cardoso VS, Mazotto AM, Supuran CT. Carbonic anhydrases from Trypanosoma and Leishmania as anti-protozoan drug targets. Bioorg Med Chem. 2017; 25: 1543-1555; b) Supuran CT. Inhibition of carbonic anhydrase from *Trypanosoma cruzi* for the management of Chagas disease: an underexplored therapeutic opportunity. Future Med Chem. 2016; 8: 311-324.

2. Pan P, Vermelho AB, Capaci Rodrigues G, Scozzafava A, Tolvanen ME, Parkkila S, Capasso C, Supuran CT. Cloning, characterization, and sulfonamide and thiol inhibition studies of an α-carbonic anhydrase from *Trypanosoma cruzi*, the causative agent of Chagas disease. J Med Chem. 2013; 56: 1761-1771.

3. a) Vermelho AB, da Silva Cardoso V, Ricci Junior E, Dos Santos EP, Supuran CT. Nanoemulsions of sulfonamide carbonic anhydrase inhibitors strongly inhibit the growth of *Trypanosoma cruzi*. J Enzyme Inhib Med Chem. 2018; 33: 139-146; b) de Menezes Dda R, Calvet CM, Rodrigues GC, de Souza Pereira MC, Almeida IR, de Aguiar AP, Supuran CT, Vermelho AB. Hydroxamic acid derivatives: a promising scaffold for rational compound optimization in Chagas disease. J Enzyme Inhib Med Chem. 2016; 31: 964-973.

4. a) Capasso C, Supuran CT. Bacterial, fungal and protozoan carbonic anhydrases as drug targets. Expert Opin Ther Targets. 2015; 19: 1689-1704; b) D Ambrosio K, Supuran CT, De Simone G. Are Carbonic Anhydrases Suitable Targets to Fight Protozoan Parasitic Diseases? Curr Med Chem. 2018; doi: 10.2174/0929867325666180326160121 (in press).

5. a) Syrjänen L, Vermelho AB, Rodrigues Ide A, Corte-Real S, Salonen T, Pan P, Vullo D, Parkkila S, Capasso C, Supuran CT. Cloning, characterization, and inhibition studies of a β-carbonic anhydrase from *Leishmania donovani chagasi*, the protozoan parasite responsible for leishmaniasis. J Med Chem. 2013; 56: 7372-7381; b) Nocentini A, Cadoni R, Dumy P, Supuran CT, Winum JY. Carbonic anhydrases from Trypanosoma cruzi and Leishmania donovani chagasi are inhibited by benzoxaboroles. J Enzyme Inhib Med Chem. 2018; 33: 286-289.

6. a) Del Prete S, Vullo D, Fisher GM, Andrews KT, Poulsen SA, Capasso C, Supuran CT. Discovery of a new family of carbonic anhydrases in the malaria pathogen Plasmodium falciparum--the η-carbonic anhydrases. Bioorg Med Chem Lett. 2014; 24: 4389-4396; b) Supuran CT, Capasso C. The η-class carbonic anhydrases as drug targets for antimalarial agents. Expert Opin Ther Targets. 2015; 19: 551-563; c) Vullo D, Del Prete S, Fisher GM, Andrews KT, Poulsen SA, Capasso C, Supuran CT. Sulfonamide inhibition studies of the η-class carbonic anhydrase from the malaria pathogen *Plasmodium falciparum*. Bioorg Med Chem. 2015; 23: 526-531; d) De Simone G, Di Fiore A,

Capasso C, Supuran CT. The zinc coordination pattern in the η-carbonic anhydrase from Plasmodium falciparum is different from all other carbonic anhydrase genetic families. Bioorg Med Chem Lett. 2015; 25: 1385-1389; e) Del Prete S, De Luca V, De Simone G, Supuran CT, Capasso C. Cloning, expression and purification of the complete domain of the η-carbonic anhydrase from *Plasmodium falciparum*. J Enzyme Inhib Med Chem. 2016; 31(sup4): 54-59.

7. a) Supuran CT. Structure and function of carbonic anhydrases. Biochem J. 2016; 473: 2023-32; b) Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nat Rev Drug Discov 2008; 7:168-81; c) Neri D, Supuran CT. Interfering with pH regulation in tumours as a therapeutic strategy. Nat. Rev. Drug Discov. 2011, 10, 767–777.

8. a) Supuran CT. Carbonic anhydrases: from biomedical applications of the inhibitors and activators to biotechnological use for CO_2 capture. J Enzyme Inhib Med Chem 2013; 28:229-30; b) Supuran CT. How many carbonic anhydrase inhibition mechanisms exist? J Enzyme Inhib Med Chem 2016; 31:345-60; c) Alterio V, Di Fiore A, D'Ambrosio K, Supuran CT, De Simone, G. Multiple binding modes of inhibitors to carbonic anhydrases: how to design specific drugs targeting 15 different isoforms? Chem Rev 2012; 112:4421-4468; d) Abbate F, Winum JY, Potter BV, Casini A, Montero JL, Scozzafava A, Supuran CT. Carbonic anhydrase inhibitors: X-ray crystallographic structure of the adduct of human isozyme II with EMATE, a dual inhibitor of carbonic anhydrases and steroid sulfatase. Bioorg Med Chem Lett. 2004; 14: 231-234; e) Capasso C, Supuran CT. An overview of the alpha-, beta-and gamma-carbonic anhydrases from Bacteria: can bacterial carbonic anhydrases shed new light on evolution of bacteria? J Enzyme Inhib Med Chem. 2015; 30: 325-332.

9. a) Supuran CT. Advances in structure-based drug discovery of carbonic anhydrase inhibitors. Expert Opin Drug Discov. 2017; 12: 61-88; b) Supuran CT, Vullo D, Manole G, Casini A, Scozzafava A. Designing of novel carbonic anhydrase inhibitors and activators. Curr Med Chem Cardiovasc Hematol Agents. 2004; 2: 49-68.

10. a) Briganti F, Mangani S, Orioli P, Scozzafava A, Vernaglione G, Supuran CT. Carbonic anhydrase activators: X-ray crystallographic and spectroscopic investigations for the interaction of isozymes I and II with histamine. Biochemistry 1997;36:10384-92; b) Clare B.W., Supuran C.T., Carbonic anhydrase activators. 3: structure-activity correlations for a series of isozyme II activators. J Pharm Sci 1994;83:768-773; c) Ilies M, Scozzafava A, Supuran CT. Carbonic anhydrase activators, in: Supuran CT, Scozzafava A, Conway J (Eds.) Carbonic Anhydrase - Its inhibitors and activators, CRC Press, Boca Raton, 2004, pp. 317-52; d) Akocak S, Lolak N, Vullo D, Durgun M, Supuran CT. Synthesis and biological evaluation of histamine Schiff bases as carbonic anhydrase I, II, IV, VII, and IX activators. J Enzyme Inhib Med Chem. 2017; 32: 1305-1312; e) Licsandru E, Tanc M, Kocsis

I, Barboiu M, Supuran CT. A class of carbonic anhydrase I - selective activators. J Enzyme Inhib Med Chem. 2017; 32: 37-46.

11. a) Temperini C, Scozzafava A, Supuran CT. Carbonic anhydrase activation and the drug design. Curr Pharm Des. 2008; 14: 708-715; b) Temperini C, Scozzafava A, Vullo D, Supuran CT. Carbonic anhydrase activators. Activation of isozymes I, II, IV, VA, VII, and XIV with l- and d-histidine and crystallographic analysis of their adducts with isoform II: engineering proton-transfer processes within the active site of an enzyme. Chemistry. 2006; 12: 7057-7066; c) Temperini C, Scozzafava A, Vullo D, Supuran CT. Carbonic anhydrase activators. Activation of isoforms I, II, IV, VA, VII, and XIV with L- and D-phenylalanine and crystallographic analysis of their adducts with isozyme II: stereospecific recognition within the active site of an enzyme and its consequences for the drug design. J Med Chem. 2006; 49: 3019-3027; d) Temperini C, Innocenti A, Scozzafava A, Supuran CT. Carbonic anhydrase activators: kinetic and X-ray crystallographic study for the interaction of D- and L-tryptophan with the mammalian isoforms I-XIV. Bioorg Med Chem. 2008; 16: 8373-8378.

12. a) Temperini C, Innocenti A, Scozzafava A, Mastrolorenzo A, Supuran CT. Carbonic anhydrase activators: L-Adrenaline plugs the active site entrance of isozyme II, activating better isoforms I, IV, VA, VII, and XIV. Bioorg Med Chem Lett. 2007; 17: 628-635; b) Temperini C, Scozzafava A, Puccetti L, Supuran CT. Carbonic anhydrase activators: X-ray crystal structure of the adduct of human isozyme II with L-histidine as a platform for the design of stronger activators. Bioorg Med Chem Lett. 2005; 15: 5136-5141; c) Temperini C, Scozzafava A, Supuran CT. Carbonic anhydrase activators: the first X-ray crystallographic study of an adduct of isoform I. Bioorg Med Chem Lett. 2006; 16: 5152-5156.

13. a) Vullo D, Nishimori I, Innocenti A, Scozzafava A, Supuran CT. Carbonic anhydrase activators: an activation study of the human mitochondrial isoforms VA and VB with amino acids and amines. Bioorg Med Chem Lett. 2007; 17: 1336-1340; b) Pastorekova S, Vullo D, Nishimori I, Scozzafava A, Pastorek J, Supuran CT. Carbonic anhydrase activators: activation of the human tumor-associated isozymes IX and XII with amino acids and amines. Bioorg Med Chem. 2008; 16: 3530-3536; c) Nishimori I, Onishi S, Vullo D, Innocenti A, Scozzafava A, Supuran CT. Carbonic anhydrase activators. The first activation study of the human secretory isoform VI. Bioorg Med Chem 2007: 15: 5351 – 5357.

14. a) Parkkila S, Vullo D, Puccetti L, Parkkila AK, Scozzafava A, Supuran CT. Carbonic anhydrase activators: activation of isozyme XIII with amino acids and amines. Bioorg Med Chem Lett. 2006; 16: 3955-3959; b) Vullo D, Innocenti A, Nishimori I, Scozzafava A, Kaila K, Supuran CT. Carbonic anhydrase activators: activation of the human isoforms VII (cytosolic) and XIV (transmembrane) with amino acids and amines. Bioorg Med Chem Lett. 2007; 17: 4107-4112; c) Vullo D, Nishimori

I, Scozzafava A, Supuran CT. Carbonic anhydrase activators: Activation of the human cytosolic isozyme III and membrane-associated isoform IV with amino acids and amines. Bioorg Med Chem Lett. 2008; 18: 4303-4307.

15. a) Supuran CT, Barboiu M, Luca C, Pop E, Brewster ME, Dinculescu A. Carbonic anhydrase activators. Part 14. Synthesis of mono- and bis- pyridinium salt derivatives of 2-amino-5-(2-aminoethyl)- and 2-amino-5-(3-aminopropyl)-1,3,4-thiadiazole, and their interaction with isozyme II. Eur J Med Chem. 1996; 31: 597-606; b) Ilies MA, Banciu MD, Ilies M, Chiraleu F, Briganti F, Scozzafava A, Supuran CT. Carbonic anhydrase activators. Part 17. Synthesis and activation study of a series of 1-(1,2,4-triazole-(1H)-3-yl)-2,4,6-trisubstituted-pyridinium salts against isozymes I, II and IV. Eur J Med Chem. 1997; 32: 911-918; c) Ilies M, Banciu MD, Ilies MA, Scozzafava A, Caproiu MT, Supuran CT. Carbonic anhydrase activators: design of high affinity isozymes I, II, and IV activators, incorporating tri-/tetrasubstituted-pyridinium-azole moieties. J Med Chem. 2002; 45: 504-510; d) Dave K, Scozzafava A, Vullo D, Supuran CT, Ilies MA. Pyridinium derivatives of histamine are potent activators of cytosolic carbonic anhydrase isoforms I, II and VII. Org Biomol Chem. 2011; 9: 2790-2800; e) Dave K, Ilies MA, Scozzafava A, Temperini C, Vullo D, Supuran CT. An inhibitor-like binding mode of a carbonic anhydrase activator within the active site of isoform II. Bioorg Med Chem Lett. 2011; 21: 2764-2768.

16. a) Canto de Souza L, Provensi G, Vullo D, Carta F, Scozzafava A, Costa A, Schmidt SD, Passani MB, Supuran CT, Blandina P. Carbonic anhydrase activation enhances object recognition memory in mice through phosphorylation of the extracellular signal-regulated kinase in the cortex and the hippocampus. Neuropharmacology. 2017; 118: 148-156; b) Supuran CT. Carbonic anhydrase activators. Future Med Chem. 2018; 10: 561-573.

17. Khalifah R.G., The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. J Biol Chem 1971; 246:2561-73

18. Carta F, Supuran CT. Diuretics with carbonic anhydrase inhibitory action: a patent and literature review (2005 - 2013). Expert Opin. Ther. Pat. 2013, 23, 681-691.

19. Masini E, Carta F, Scozzafava A, Supuran CT. Antiglaucoma carbonic anhydrase inhibitors: a patent review. Expert Opin. Ther. Pat. 2013, 23, 705-716.

20. a) Scozzafava A, Supuran CT, Carta F. Antiobesity carbonic anhydrase inhibitors: a literature and patent review. Expert Opin. Ther. Pat. 2013, 23, 725-735; b) Supuran CT. Carbonic Anhydrases and Metabolism. Metabolites. 2018; 8: E25.

21. a) Monti SM, Supuran CT, De Simone G. Anticancer carbonic anhydrase inhibitors: a patent review (2008 - 2013). Expert Opin. Ther. Pat. 2013, 23, 737-749; b) Supuran CT. Carbonic Anhydrase Inhibition and the Management of Hypoxic Tumors. Metabolites. 2017, 7, E48; c) Ward

C, Langdon SP, Mullen P, Harris AL, Harrison DJ, Supuran CT, Kunkler IH. New strategies for targeting the hypoxic tumour microenvironment in breast cancer. Cancer Treat Rev. 2013; 39: 171-179; d) Garaj V, Puccetti L, Fasolis G, et al. Carbonic anhydrase inhibitors: novel sulfonamides incorporating 1,3,5-triazine moieties as inhibitors of the cytosolic and tumour-associated carbonic anhydrase isozymes I, II and IX. Bioorg Med Chem Lett. 2005; 15: 3102-3108; e) Casey JR, Morgan PE, Vullo D, Scozzafava A, Mastrolorenzo A, Supuran CT. Carbonic anhydrase inhibitors. Design of selective, membrane-impermeant inhibitors targeting the human tumor-associated isozyme IX. J Med Chem. 2004; 47: 2337-2347.

22. a) Supuran CT. Carbonic anhydrase inhibition and the management of neuropathic pain. Expert Rev Neurother. 2016; 16: 961-968; b) Di Cesare Mannelli L, Micheli L, Carta F, Cozzi A, Ghelardini C, Supuran CT. Carbonic anhydrase inhibition for the management of cerebral ischemia: in vivo evaluation of sulfonamide and coumarin inhibitors. J Enzyme Inhib Med Chem. 2016; 31: 894-899.

23. a) Margheri F, Ceruso M, Carta F, Laurenzana A, Maggi L, Lazzeri S, Simonini G, Annunziato F, Del Rosso M, Supuran CT, Cimaz R. Overexpression of the transmembrane carbonic anhydrase isoforms IX and XII in the inflamed synovium. J Enzyme Inhib Med Chem. 2016; 31(sup4): 60-63; b) Bua S, Di Cesare Mannelli L, Vullo D, Ghelardini C, Bartolucci G, Scozzafava A, Supuran CT, Carta F. Design and Synthesis of Novel Nonsteroidal Anti-Inflammatory Drugs and Carbonic Anhydrase Inhibitors Hybrids (NSAIDs-CAIs) for the Treatment of Rheumatoid Arthritis. J Med Chem. 2017; 60: 1159-1170.

24. a) Vullo D, De Luca V, Scozzafava A, Carginale V, Rossi M, Supuran CT, Capasso C. The first activation study of a bacterial carbonic anhydrase (CA). The thermostable α-CA from Sulfurihydrogenibium yellowstonense YO3AOP1 is highly activated by amino acids and amines. Bioorg Med Chem Lett. 2012; 22: 6324-6327; b) Innocenti A, Zimmerman SA, Scozzafava A, Ferry JG, Supuran CT. Carbonic anhydrase activators: activation of the archaeal beta-class (Cab) and gamma-class (Cam) carbonic anhydrases with amino acids and amines. Bioorg Med Chem Lett. 2008; 18: 6194-6198; c) Vullo D, Del Prete S, Osman SM, Alasmary FAS, AlOthman Z, Donald WA, Capasso C, Supuran CT. Comparison of the amine/amino acid activation profiles of the β- and γ-carbonic anhydrases from the pathogenic bacterium *Burkholderia pseudomallei*. J Enzyme Inhib Med Chem. 2018; 33: 25-30; d) Vullo D, Del Prete S, Osman SM, AlOthman Z, Capasso C, Donald WA, Supuran CT. *Burkholderia pseudomallei* γ-carbonic anhydrase is strongly activated by amino acids and amines. Bioorg Med Chem Lett. 2017; 27: 77-80; d) Angeli A, Alasmary FAS, Del Prete S, Osman SM, AlOthman Z, Donald WA, Capasso C, Supuran CT. The first activation study of a δ-carbonic anhydrase: TweCAδ from the diatom *Thalassiosira weissflogii* is effectively activated by amines and amino acids. J Enzyme Inhib Med Chem. 2018; 33: 680-685.

25. a) Stefanucci A, Angeli A, Dimmito MP, et al. Activation of β- and γ-carbonic anhydrases from pathogenic bacteria with tripeptides. J Enzyme Inhib Med Chem. 2018; 33: 945-950; b) Angeli A, Donald WA, Parkkila S, Supuran CT. Activation studies with amines and amino acids of the β-carbonic anhydrase from the pathogenic protozoan *Leishmania donovani chagasi*. Bioorg Chem. 2018; 78: 406-410.